IDENTIFICATION OF A PRECURSOR FOR ONE OF THE SEMLIKI FOREST VIRUS MEMBRANE PROTEINS

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1. Introduction

We are studying the envelope of Semliki forest virus (SFV) as a model for membrane structure and biogenesis. Semliki forest virus and other group A arboviruses acquire their envelopes by budding through the host cell plasma membrane [1]. The viral envelope resembles the host cell plasma membrane in its lipid composition [2], but has a much simpler protein composition: one protein with an apparent molecular weight of about 50,000 has been found in the membrane [3]. In Sindbis virus this protein has recently been split into two bands (E_1 and E_2) using discontinuous sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis [4]. Both of these polypeptides contain carbohydrate.

Studies on SFV protein formation are facilitated by the fact that virus infection effectively shuts off host cell protein synthesis [5]. Attempts to find out whether the structural polypeptides of the virus are translated as primary gene products or in precursor form(s) have so far been inconclusive [6–9]. Our previous studies have suggested a precursor role for a non-structural glycopolypeptide (NSP68) which is found in extracts of infected cells [10]. This polypeptide has an apparent molecular weight of 68,000 in 5% acrylamide SDS-gels. Here we show that SFV contains two envelope polypeptides, E_1 and E_2, and that these have different primary structures. We also show that NSP68 is a precursor for the envelope polypeptide E_2, which is confirmed by the isolation of a temperature-sensitive mutant of SFV, in which precursor cleavage is blocked.

2. Materials and methods

A prototype SFV strain, unlabelled, or labelled with [^{35}S]-methionine (Radiochemical Centre, Amersham), was grown in BHK21 cells and purified as described previously [11]. Polyacrylamide gel electrophoresis was performed in two ways, either using the continuous system described by Weber and Osborn [12] or the discontinuous system of Neville [13] with a spacer gel using a lower gel buffer with pH of 9.18. The former gave better separation between NSP68 and the envelope polypeptides, both of which here migrated together, whereas the latter enabled us to separate the envelope polypeptides from each other. Prior to electrophoresis the samples were treated with 1% SDS and 1% 2-mercaptoethanol at 90° for 30 min. The gels were either stained with Coomassie blue or sliced longitudinally, dried and autoradiographed as described by Fairbanks et al. [14]. In the pulse-chase experiments the gels were cut into 2 mm slices and the radioactivity determined in a toluene based scintillation fluid after NCS-treatment [10].

In the fingerprinting studies, the [^{35}S]-methionine polypeptide to be eluted from the SDS-gel was localized by autoradiography of the centre slice [14]. The corresponding region was cut out and eluted in 0.1% SDS, 0.05 M ammonium bicarbonate overnight at 37°. To proceed through the next steps at least 5×10^5 ^{35}S cpm per gel region were necessary. 0.5 mg bovine serum albumin was added to the samples which were lyophilized, performic acid oxidized [15] and lyophilized again. SDS was removed as described by Weber and Kuter [16]. The protein was then digested with trypsin and chymotrypsin (50 μg of each) in 0.1 M ammonium bicarbonate for 16 hr at 37°. The resulting

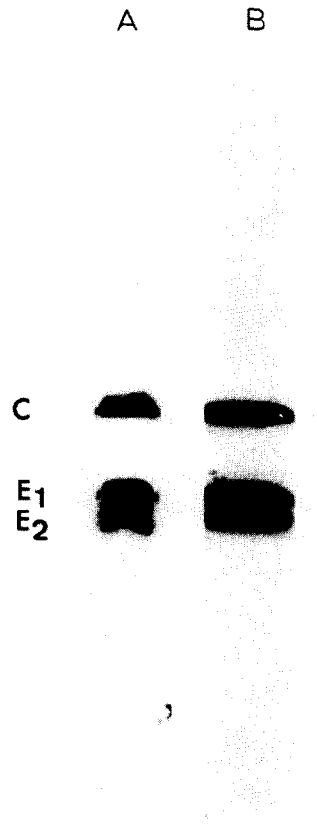

Fig. 1. Discontinuous 11% polyacrylamide SDS-gel electrophoresis [13] of SFV. A) Autoradiogram of a gel to which [^{35}S]-methionine labelled SFV (3×10^{6} cpm) was applied. B) Stained with Coomassie blue, 30 μg of SFV protein applied. Migration upwards.

radioactive peptides were fractionated on Whatman 3M paper by chromatography (n-butanol–acetic acid–H_2O, 17:5:25, v/v) followed by electrophoresis at pH 3.5 in the second dimension. The fingerprints were autoradiographed for 1–3 weeks.

The temperature-sensitive mutant ts-1 of SFV was obtained by treatment with nitrosoguanidine and will be described in detail elsewhere (Keränen and Kääriäinen, in preparation). Pulse-chase experiments were performed in BHK21 cells and chick embryo fibroblasts at 27°, 37° and 39°. The infected cells were pulsed with [^{14}C]-amino acids, [^{3}H]-amino acids or with [^{35}S]-methionine as indicated in the figure legends and chased with an excess of the corresponding unlabelled amino acids. Cytoplasmic extracts were prepared as described previously [10].

3. Results and discussion

Fig. 1 shows that, in addition to the nucleocapsid polypeptide C, which has a molecular weight of 34,000 [3], two closely spaced bands E_1 and E_2 can be demonstrated both by Coomassie blue staining and autoradiography of gels containing [^{35}S]-methionine labelled SFV. The nucleocapsid and the envelope polypeptides E_1 and E_2 were identified after separation of the nucleocapsid from the envelope as described previously [11]. Fingerprints were made to find out whether E_1 and E_2 are indeed two different polypeptides. They (together with C) were isolated from [^{35}S]-methionine SFV and digested with trypsin and chymotrypsin. The autoradiograms shown in fig. 2A–C are clearly different.

The role of NSP68 was studied by pulse-chase experiments and fingerprinting. A cytoplasmic extract of chick embryo fibroblasts infected with the ts-1 mutant at 39° (the restrictive temperature) was prepared after a pulse of [^{3}H]-amino acids. This was mixed with the cytoplasmic extract from wild-type infected cells pulsed similarly with [^{14}C]-amino acids. SDS-electrophoresis showed that NSP68 was present in both cell extracts (fig. 3A). The functions of the proteins with slower electrophoretic mobility than NSP68 found in the ts-1 infected cells are not known [6–8].

After chasing with unlabelled amino acids the radioactivity in NSP68 was significantly reduced in cells infected with wild-type SFV and a corresponding increase was seen in the envelope polypeptides (E). No such change was observed in cells infected with the mutant (fig. 3B).

Discontinuous SDS-polyacrylamide gels show that E_2 is almost absent from the cells infected with the wild-type and the mutant virus after 15 min labelling with [^{35}S]-methionine, whereas both the nucleocapsid polypeptide C and the envelope polypeptide E_1 are already formed (fig. 4). Radioactivity appears in E_2 during the subsequent chase period in cells infected with wild-type SFV but not in those infected with the ts-1 mutant. At the permissive temperature (27°) the kinetics of labelling of NSP68 and E_2 are the same in both ts-1 and wild-type infected cells.

Further evidence for the precursor role of NSP68 was obtained by fingerprinting. Fig. 2 shows that the trypsin–chymotrypsin fingerprint of [^{35}S]-methionine labelled NSP68 is similar to that obtained for E_2. Some

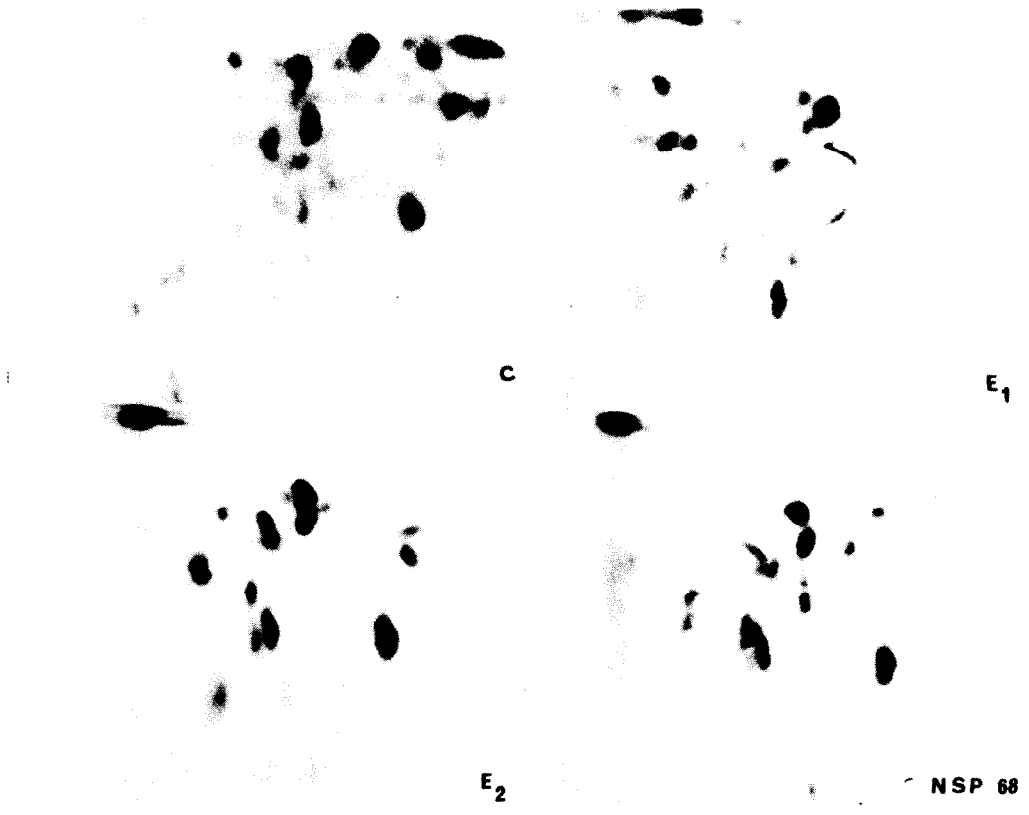

Fig. 2. Autoradiograms of fingerprints of nucleocapsid (C) and envelope (E_1 and E_2) polypeptides from [^{35}S]-methionine labelled SFV, and of the nonstructural polypeptide (NSP68) from chick embryo fibroblasts infected with SFV mutant ts-1 at 39°. C, E_1 and E_2 were located by autoradiography of the centre slice of a gel similar to that in fig. 1B. NSP68 was located in a similar fashion from continuous SDS-gels [12] in which a cell extract similar to that applied to gel 3 in fig. 4 had been electrophoresed. The trypsin–chymotrypsin digests were applied to the top left corner of the paper, chromatography was downwards and electrophoresis to the right.

additional peptides are seen in NSP68 but further purification of NSP68 is needed to confirm their presence. These results clearly show that NSP68 functions as a precursor for E_2. Similar results have been obtained with Sindbis virus (S. Schlesinger, personal communication).

The segment that is cleaved from NSP68 has not yet been identified. We have observed a glycopeptide in cells infected with wild-type SFV [10]. This peptide has an apparent molecular weight of about 20,000 in 7.5% acrylamide SDS-gels, and may be the "missing" piece. It is absent from infected cells which have been treated with canavanine, an arginine analogue, which arrests virus formation [10]. Canavanine inhibits glycosylation of the virus polypeptides and cleavage of NSP68.

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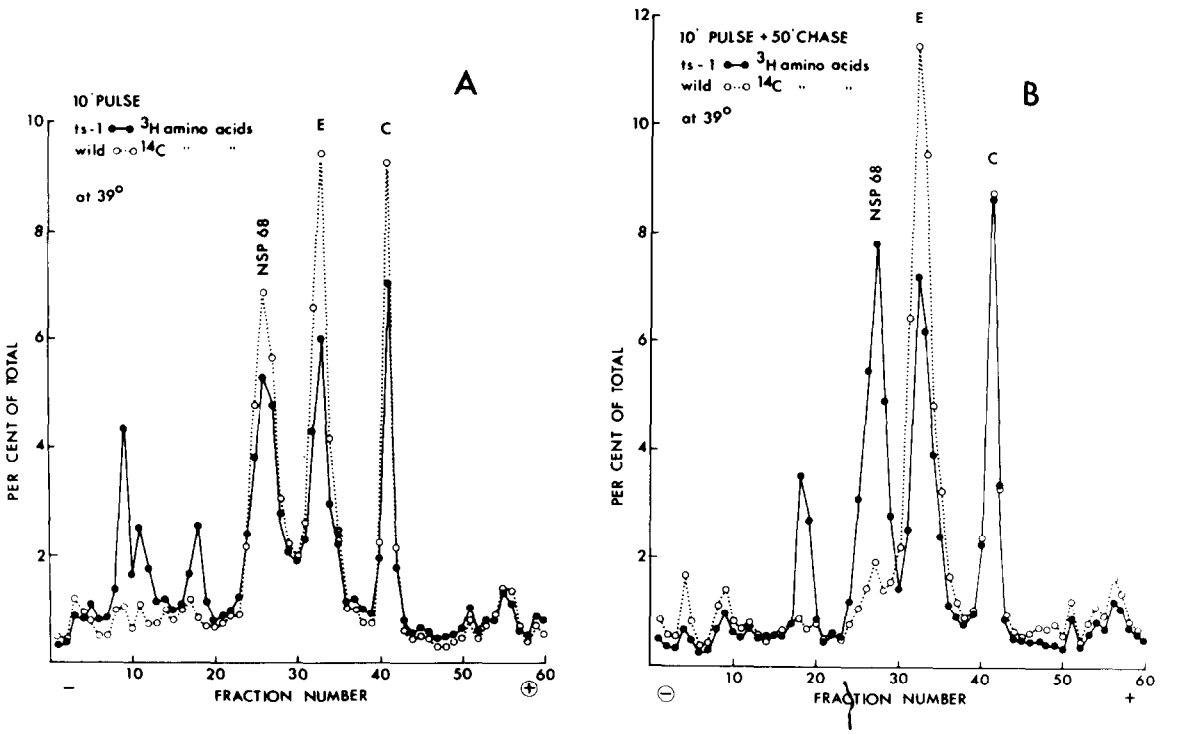

Fig. 3. Continuous 5% polyacrylamide SDS-gel electrophoresis [12] of SFV proteins synthesized in chick embryo fibroblasts 5 hr after infection at 39°. Cells infected with wild-type SFV were pulsed with 20 μCi [^{14}C]-amino acids (6.7 μCi/ml) for 10 min and chased with an excess of unlabelled amino acids for 50 min. Cells infected with mutant ts-1 similarly pulsed with 100 μCi [^{3}H]-amino acids (33 μCi/ml) and chased. The wild-type and ts-1 infected cytoplasmic extracts were mixed prior to electrophoresis. A) Pulse without chase. B) Pulse with chase. 22,000 ^{14}C dpm and 94,000 ^{3}H dpm to gel B. ^{14}C (o--o--o). ^{3}H (●—●—●). Migration to the right.

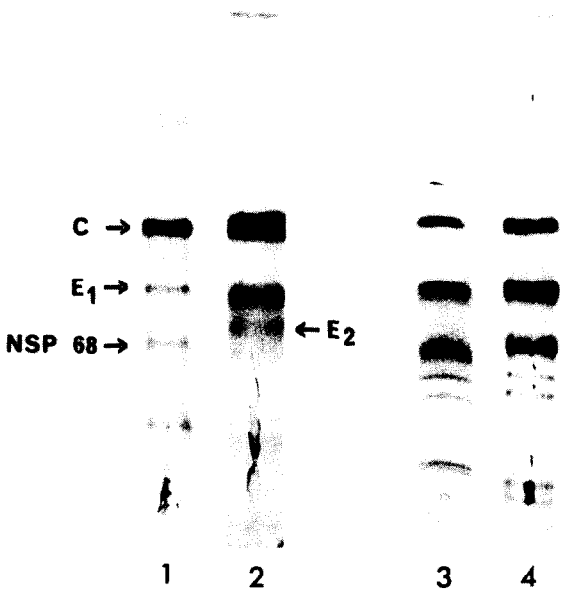

Fig. 4. Discontinuous 11% polyacrylamide SDS-gel electrophoresis [13] of SFV proteins synthesized in cells 5 hr after infection. BHK21 cells were infected with wild-type SFV at 37° (gels 1 and 2). Chick embryo fibroblasts were infected with mutant ts-1 at 39° (gels 3 and 4). Gels 1 and 3: Pulse with 2 mCi [^{35}S]-methionine (0.33 mCi/ml) for 15 min. Gels 2 and 4: As gels 1 and 3 plus a 45 min chase with excess unlabelled methionine. Aliquots of the cytoplasmic extracts were electrophoresed and autoradiographed. Migration upwards.

References

[1] W.K. Joklik and H.J. Zwerrink, Ann. Rev. Genetics 5 (1971) 297.
[2] O. Renkonen, C.G. Gahmberg, K. Simons and L. Kääriäinen, Virology 46 (1971) 318.
[3] K. Simons and L. Kääriäinen, Biochem. Biophys. Res. Commun 38 (1970) 981.
[4] M.J. Schlesinger, S. Schlesinger and B.W. Burge, Virology 47 (1972) 539.
[5] R.M. Friedman, J. Virol. 2 (1968) 26.
[6] C.J. Burrell, E.M. Martin and P.D. Cooper, J. Gen. Virol. 6 (1970) 319.
[7] A. Igarashi, Biken J. 13 (1970) 289.
[8] E.R. Pfefferkorn and M.K. Boyle, J. Virol 9 (1972) 187.
[9] R.M. Friedman, Biochem. Biophys. Res. Commun. 37 (1967) 369.
[10] M. Ranki, L. Kääriäinen and O. Renkonen, Acta Pathol. Microbiol. Scand., in press.
[11] L. Kääriäinen and H. Soderlund, Virology 43 (1971) 291.
[12] K. Weber and M. Osborn, J. Biol. Chem. 244 (1969) 4406.
[13] D.M. Neville, J. Biol. Chem. 246 (1971) 6328.
[14] G. Fairbanks, C. Levinthal and R.H. Reeder, Biochem. Biophys. Res. Commun. 20 (1965) 393.
[15] C.H.W. Hirs, in: Methods in Enzymology, Vol. 11, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York, 1967) p. 197.
[16] K. Weber and D.J. Kuter, J. Biol. Chem. 246 (1971) 4504.